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# Generation and Characterization of VEGF-E-Mutants

## Diploma Thesis

by

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# 1 ABSTRACT

Vascular endothelial growth factors constitute a family of polypeptides, VEGF-A, -B, -C, -D and PlGF, that regulate blood and lymphatic vessel development upon binding to three types of receptors, VEGFR-1 -3. Pox viruses of the Orf family encode highly related proteins called VEGF-E that show only 25 % amino acid identity with VEGF-A but bind with almost the same affinity to VEGFR-2. The crystal structure studies showed distinct conformational differences between VEGF-E and the other VEGF's in the loops L1 and L3 which presumably determine receptor specificity. To further investigate the determinants of receptor specificity selected segments in L1 and L3 between VEGF-E and PlGF were exchanged. The mutated proteins were produced in the yeast *Pichia pastoris* and purified over nickel affinity and size exclusion chromatography. Binding assays on PAE cells expressing either VEGFR-1 or -2 confirmed that these loops determine receptor specificity. The loop chimera of VEGF-E where both the loops L1 and L3 were exchanged showed specific binding to VEGFR-1 and was crystallized. However, crystal structure determination was not possible due to poor crystal quality.
Moreover, preliminary expression tests of selected VEGFR-2 domains in *E.coli* failed to provide protein of suitable quality for future crystallographic studies of ligand-receptor complexes.

# 2 INTRODUCTION

## 2.1 Angiogenesis

Angiogenesis, or formation of new blood capillaries from preexisting vessels, is an important biological process and plays both beneficial and detrimental roles in the organism. Physiological angiogenesis occurs during embryogenesis, the female reproductive cycle and in wound healing. Abnormally enhanced angiogenic response is observed in rheumatoid arthritis, diabetic retinopathy, and during tumor development.

Inhibition of the neovasculature was shown to abolish or slow tumor growth in various experimental models. On the other hand, promotion of the angiogenic response can prove beneficial in the treatment of ischemic conditions, such as myocardial ischemia/ infarction. It is therefore obvious, that the ability to prevent and treat many of these diseases relies on our understanding of the molecular mechanisms underlying the angiogenic response.

Angiogenesis is a result of complex interplay of positive and negative regulators. The VEGFs (Vascular Endothelial Growth Factors) are molecules that serve as inducers of angiogenesis and therefore it is important to know more about the interplay of these molecules and their receptors. (Petrova *et al*., 1999)

## 2.2 VEGF's and VEGF receptors

There is a consensus that the Vascular Endothelial Growth Factors (VEGFs) are crucial for vascular development and neovascularization in physiological and pathological processes in both embryo and adult. More recent data indicate essential roles for the VEGFs in haematopoietic cell function and in lymph angiogenesis. VEGF has also been implicated in pathological angiogenesis associated with tumors, intraocular neovascular disorders and other conditions. VEGF denotes a family of homodimeric glycoproteins, which currently consists of five mammalian members VEGF-A, -B, -C, -D and placenta like growth factor (PlGF), Orf virus-encoded VEGF-like proteins called VEGF-E and a series of snake venoms collectively called VEGF-F. Due to alternative splicing or posttranslational processing, PlGF, VEGF-A, -B, -C and –D are generated in a number of functionally distinct isoforms. The biological function of VEGF is mediated through binding to three tyrosine kinase receptors, VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1) and VEGFR-3 (Flt-4) that are localized on the cell surface of endothelial cells, in bone-marrow derived hematopoietic precursor cells and in malignant cells. VEGFs bind, in an overlapping pattern, to the different VEGF receptor tyrosine kinases (Figure 1). The role of each receptor-ligand interaction remains to be precisely defined but knockout studies indicate that VEGFR-1 plays different roles, negative and regulatory, in angiogenesis. VEGFR-2 is the main signal-transducing VEGF receptor for mitogenesis of endothelial cells and VEGFR-3 is involved in angiogenesis of the lymphatic vasculature. (Cross *et al*., 2003)

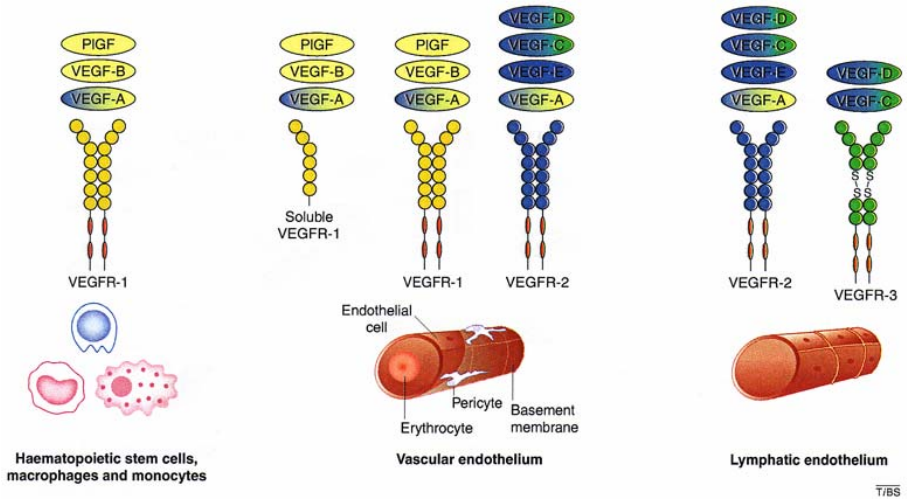


**Figure 1.** *Schematic illustration of vascular endothelial growth factor receptor expression patterns and ligand specificity*. The VEGF ligand family includes VEGF-A, -B, -C, -D, -E and PlGF, which all bind in a specific manner to three different receptor tyrosine kinases, VEGFR-1, -2 and -3. VEGFR-1 and its ligands are yellow, VEGFR-2 and its ligands are blue, and VEGFR-3 and its ligands are green. Ligands shaded in two colors bind to more than one receptor type. Both PlGF and VEGF-B are selective ligands for VEGFR-1, whereas VEGF-E is a selective ligand for VEGFR-2. In contrast, all VEGF-A isoforms bind to both VEGFR-1 and VEGFR-2.

The VEGF receptors 1-3 belong to the class III receptor tyrosine kinases of the platelet-derived growth factor (PDGF) receptor subfamily which is characterized by the presence of seven immunoglobulin homology domains in their extracellular, ligand-binding part and an intracellular tyrosine kinase domain split by a kinase insert. Ligand binding is thought to induce homodimerization of the VEGF receptors and transphosphorylation of their intracellular domains, which allows the activation of complex intracellular signaling pathways. The VEGF molecule represents an antiparallel disulfide-linked homodimer which interacts with the receptor via binding sites at the poles of each molecule. As a result of domain deletion studies of VEGFR-1 and VEGFR-2 it was shown that ligand binding function is localized to the second and third Ig domain, but the two domains play different roles in the VEGFR-1 and VEGFR-2 interaction with the VEGFs. (Ferrara *et al*, 2003; Petrova *et al*., 1999)

## 2.3 VEGFR-2 and its ligand VEGF-E

To clarify the biological functions driven by each VEGFR one needs receptor specific proteins. Orf viruses encode for a VEGF-like protein that specifically binds at high affinity and activates VEGFR-2. Orf viruses, the type species of the parapoxvirus genus, belong to the parapoxvirus family and produce a postular dermatitis in sheep, goats and humans. Several reports have described prominent vascular changes associated with lesions caused by Orf virus. Lesions produced after infection show extensive proliferation of vascular endothelial cells, dilation of blood vessels and dermal swelling. This is apparently due to the fact, that Orf virus encodes a homolog of mammalian vascular endothelial growth factor (VEGF-A).
The proteins encoded by these sequences show an extraordinary degree of sequence variation and are collectively called VEGF-E. They have been found in more than 20 independent parapoxvirus isolates. The coding sequence for these proteins was apparently captured from a mammalian host and the sequence variation reflects numerous cycles of mutation and genetic recombination. VEGF-E family members show only 20-25% amino acid identity with VEGF-A but bind with almost the same affinity as VEGF-A to VEGFR-2. VEGF-E ligands lack a heparin-binding domain yet retain binding to neuropilin, a co-receptor for VEGFRs that is essential for proper vascular development. VEGF-E variants are potent mitogens stimulating proliferation of human endothelial cells in vitro and vascularization of sheep skin in vivo with potencies equivalent to VEGF-A. (Wise *et al*., 2003)
Structural studies of the receptor binding domains of VEGF-A and PlGF in their free and receptor-bound form have been reported previously and the crystal structures of these ligands in complex with the minimal ligand binding fragment (Ig-like domain 2) of VEGFR-1 are known. (Christinger *et al*, 2003; Iyer, S. *et al*, 2001; Muller, Y. A. *et al*, 1997; Starovasnik, M. A. *et al*, 1999; Wiesmann, C., *et al*, 1997)
Recently, Pieren and co-workers at PSI determined the crystal structure of VEGF-E (NZ2 variant). (Pieren *et al*., 2005)
The structure of VEGF-E Nz2 revealed high similarity to the known structural homologs VEGF-A, PlGF and the snake venoms Vammin and VR-1, which are all dimeric and fold into the common cystein knot structure. Distinct conformational differences were observed in the loops L1 and particularly in L3 which presumably determine receptor specificity (Figure 2).

Loop-3 of VEGF-E contains a highly flexible GS-rich motif that differs from L3 in all other structural homologs. However, none of these structural features are exclusively shared with the other receptor 2 specific ligands.

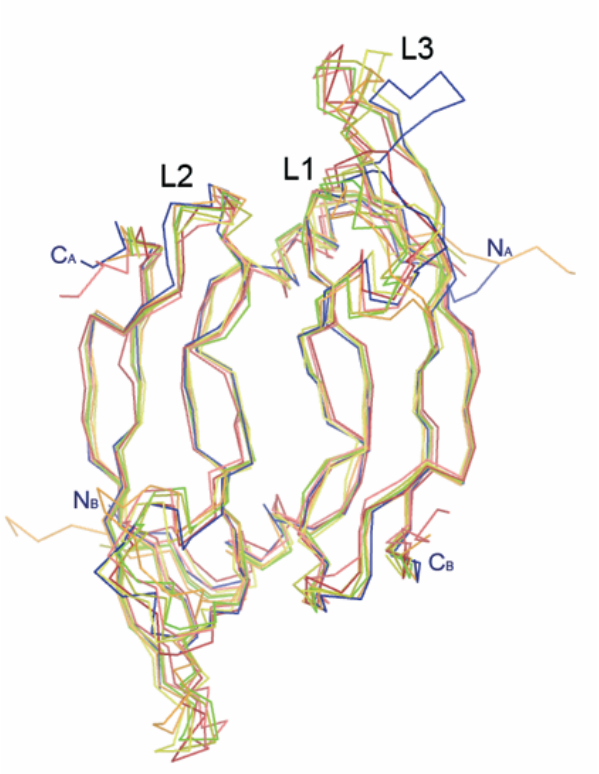


**Figure 2.** *Comparison of VEGF-E with other structural homologs.* Superposition in stereo of the $C_\alpha$-traces (112 Cα atoms) of VEGF-A (green), VEGF-A-D2 (olivegreen), PlGF (orange), PlGF-D2 (yellow), Vammin (red), VR-1 (salmon) and VEGF-E NZ2 (blue). In VEGF-E loop L3 is twisted away from all other observed conformations.

Mutation studies with VEGF-A showed that single amino acids in these loops are essential for the binding of VEGF-A to VEGFR-2. Mutations of these amino acids give rise to much weaker interactions. Although these residues are not conserved in VEGF-E it binds to VEGFR-2 with high affinity.

Due to structural comparison and binding assays with different mutants it is supposed that the molecular basics of the binding and specifity of the VEGF's to the different receptors lie in the structural parameters of loop-1 and loop-3.

## 2.4 Goal of the diploma thesis

The comparison of the crystal structures of PlGF, VEGF-A and VEGF-E showed that the distinct conformational differences are in loop-1 and loop-3. It has to be answered if these are critical determinants for receptor affinity and if this might be the reason why VEGF-E only binds to VEGFR-2. Therefore I created different VEGF-E loop mutants during my semester thesis. In two constructs I replaced either loop-1 or loop-3 by the one from PlGF, which only binds to VEGFR-1, and in a third construct, I changed both of this loops with the ones of PlGF. Finally, I inserted a point mutation in loop-1 of a wildtype VEGF-E, so that the DNA sequence encodes no longer for the amino acid arginine but for isoleucine. Arginine 46 is able to form a salt bridge to glutamate 64 in loop-2. This salt bridge eventually blocks the gateway to VEGFR-1. I succeeded in generating three of these four mutants.
During the semester thesis arose the goal for my diploma work. The aim was to generate and characterize the fourth of this VEGF-E mutant, the $L_{13}$-mutant. The practical work consisted in cloning, protein expression in *Pichia pastoris* and purification by nickel affinity chromatography and gel filtration. The other three mutants have already been produced by someone else in the lab.
Moreover, functional assays with this purified proteins were planned to show how the different mutations affect the binding of VEGF-E to VEGFR-1 or -2.
Finally, depending on the outcome of the binding assays, the most important mutants had to be crystallized. The structure determination should reveal more details about the conformational change that might occur through the mutations.

As an option, a selected VEGFR-2 fragment, which contains the minimal binding domains for VEGF-A and –E, could be cloned for expression in *E.coli*. The aim of this additional project was to speed up the production of functional VEGFR-2 domains for future crystallographic studies of ligand-receptor complexes.

# 3 MATERIALS AND METHODS

## 3.1 VEGF-E-$L_{13}$-Mutant

### 3.1.1 Cloning of VEGF-E-$L_{13}$-Mutant by Site Directed Mutagenesis

**Table 1** Scheme for the PCR Reaction

| | **Sample Reaction** | **Positive Control Reaction** | **Negative Control Reaction** |
|---|---|---|---|
| template (expression clone) 30 ng | 0.3 µl (VEGF-E-$L_1$) | 0.5 µl (VEGF-$A_{121}$) | 0.5 µl (VEGF-$A_{121}$) |
| primer forward F3 (1:10) 125 ng | 1 µl | 1 µl (5'AOX) | -- |
| primer reverse R3 (1:10) 125 ng | 1 µl | 1 µl (3'AOX) | -- |
| dNTPs [10 mM] | 1 µl | 1 µl | 1 µl |
| buffer 5x F-518 (FINNZYMES) | 10 µl | 10 µl | 10 µl |
| $H_2O$ | 35 µl | 35 µl | 37 µl |
| polymerase phusion (F-530L, FINNZYMES) | 1 µl | 1 µl | 1 µl |
| **Total** | **50 µl** | **50 µl** | **50 µl** |

A sequenced VEGF-E-$L_1$-mutant construct (PCR Gateway® Technology: expression clone) that contains loop-1 of PlGF was used as template for the sample reaction. This mutant is a chimera of VEGF-E and loop-1 of human PlGF.

The primers (F3; R3) are specific for this reaction and have the following sequences (Microsynth):

Forward primer, F3:
5'- GTAAACGTGAGCATGGAACTCCTG**AAGATCCGTTCTGGGGACCGG**AACGGGATGCAACGTCTGAGC-3'
82 K I R S G D R 88
$T_M$ 56.7°C 58.7°C

Reverse primer, R3
5'- GCTCAGACGTTGCATCCCGTT**CCGGTCCCCAGAACGGATCTT**CAGGAGTTCCATGCTCACGTTTAC-3'
$T_M$ 58.7°C 56.7°C

**Table 2** PCR-Conditions

| **Segment** | **Cycles** | **Temperature** | **Time** |
|---|---|---|---|
| **1** | 1 | 98°C | 2'30" |
| **2** | 20 | 98°C | 30" |
| | | 62°C | 30" |
| | | 72°C | 2'30" |
| | hold | 10°C | |

After the PCR 1 µl of the enzyme DpnI (Fermentas; 10 u/µl, 2500 u) was added to the samples and the mix was incubated for 3 hours at 37° C. This digest was done to degrade the template DNA.
The samples were then purified by MinElute PCR Purification Kit and eluted in 15 µl water.

### 3.1.2 Electroporation in *E.coli*

100 µl cells (Top10, electrocompetent) were thawed on ice and mixed with 400 µl of 10 % glycerol. 60 µl of this cell suspension were mixed with 4 µl of purified DNA and immediately electroporated using the following conditions: 200 Ohm, 1.7 kV, 25 uF, which gave about 4.0-4.5 ms pulse time. Immediately after pulse 400 µl low salt LB media were added and the mixture was transferred into a 5 ml Snap cap. Upon 1 hour incubation at 37° C by shaking at 200 rpm, 150 µl culture were spreaded on low salt LB Agar plates with Zeocin™ [25 µg/ml] and incubated over night at 37° C.

### 3.1.3 Miniculture and Miniprep

Miniculture:

Single colonies were picked from the plates with a sterile toothpick to inoculate over night cultures in 5 ml low salt LB media with Zeocin™ [25 µg/ml] at 37° C and 200 rpm.

Miniprep:

The over-night cultures were centrifuged for 10 min at 4° C and 3000 xg (Eppendorf Centrifuge 5810R). The supernatant was discarded and the pellets resuspended in 300 µl cold buffer P1 (50 mM Tris pH 8, 10 mM EDTA, 0.1 mg/ml RNase A). 300 µl of buffer P2 (200 mM NaOH, 1% SDS) were added and the mixture was incubated for 5 min at RT. Upon addition of 300 µl of cold buffer P3 (3M $KCOOCH_3$, pH 5.5) the mixture was incubated for 10 min on ice and subsequently centrifuged for 10 min at 4° C and 11'000 xg (Eppendorf Centrifuge 5415R). The supernatant, which contained the plasmid in solution, was transferred to new 2 ml-tubes. 700 µl isopropanol (100%) were then added and the mixture was incubated for 5–10 min at RT before centrifugation for 15 min at RT and 11'000 xg (Eppendorf Centrifuge 5415R). The supernatant was discarded and the pellet (plasmid DNA) was washed twice with 70% ethanol. Finally the pellet was dried and resuspended in 40 µl $H_2O$.

### 3.1.4 Restriction Digest

The samples were digested with the enzymes Afl III (New England Bio Labs, 5 u/µl) and Eco24I (Fermentas; 10 u/µl, 1500 u). The following buffers were used: for Afl III buffer H (SuRE Cut Buffer H for Restriction Enzymes, 10x conc., Roche) and for Eco 24I buffer B (SuRE Cut Buffer B for Restriction Enzymes, 10x conc., Roche).

**Table 3** Scheme for the restriction digest

| | |
|---|---|
| restriction enzyme | 0.5 µl |
| buffer | 1 µl |
| H2O | 4.5 µl |
| DNA | 4 µl |
| **Total** | **10 µl** |

The samples were incubated at 37° C for 2 hours and analyzed on a 1 %-agarose gel.

### 3.1.5 EasyComp™ Transformation into *Pichia pastoris*

For transformation into *P. pastoris* the plasmid DNA had to be linearized.

**Table 4** Linearization of the plasmid DNA (restriction digest)

| | |
|---|---|
| DNA (7 µg/µl) | 10 µl |
| buffer Sac I+ (SuRE Cut Buffer A for Restriction Enzymes, 10x conc., Roche) | 15 µl |
| Sac I (Fermentas 10 u/µl, 1200 u) | 5 µl |
| $H_2O$ | 120 µl |
| **Total** | **150 µl** |

3 µg of linearized *Pichia* expression vector DNA was added to 50 µl of competent cells (freshly prepared or frozen competent *Pichia* cells (X-33)). 1 ml of Solution II was added to the DNA/cell mixture and mixed by flicking the tubes. The transformation reaction was then incubated for 1 hour at 30° C, and mixed every 15 min by flicking the tubes. After heat shock at 42° C for 10 min, the cells were splited into 2 microcentrifuge tubes and 1 ml of YPD medium was added to each tube. The cells were further incubated at 30° C for 1 hour to allow expression of Zeocin™ resistance. The samples were then pelleted by centrifugation at 3000 xg for 5 min at RT and the supernatant was discarded. Each tube of cells was resuspended in 500 µl of Solution III and the cells were combined into one tube. Again the cells were pelleted by centrifugation at 3000 g for 5 min at RT and the supernatant was discarded. The pellets were resuspended in 100 µl of Solution III and spreaded on low salt LB agar plates with Zeocin™ [100 µg/ml].

### 3.1.6 Protein expression

Small-scale expression
Small-scale expression was performed to select for high-expression clones. Positive clones were inoculated into 5 ml YPD media containing Zeocin™ [100 µg/ml] for a first biomass accumulation. After two days incubation in a shaking incubator with 200 rpm at 30° C, the cells were harvested by centrifugation at 3000 xg (Eppendorf Centrifuge 5810R) for 5 min at RT. The pellet was then resuspended in 100 ml BMGY media and further incubated at 30°C in the shaking incubator for another day, so that the cultures reached an $OD_{600}$ = 10. The cells were harvested and the pellets were resuspended in 100 ml BMMY media to induce expression. 100 % methanol was added to a final concentration of 2 % methanol every 24 hours to maintain induction. 1 ml samples of each culture were taken at time points 0, 24, 48 and 72h post induction to analyze expression levels and determine the optimal time post induction to harvest the supernatant. Protein expression was detected with Coomassie-stained SDS-PAGE.

Large-scale expression
50 ml of YPD media with Zeocin™ [100 µg/ml] were inoculated with the test expressing clones and grown in a shaking incubator at 30° C for two days. The procedure then was the same as described above but with 1000 ml media instead of 100 ml. 72 h of induction turned out as the optimal time point to harvest the protein.

### 3.1.7 Preparation of deglycosylated VEGF-E-mutants

Expression was stopped by centrifugation of the cell culture in 1000 ml bottles for 20 min at 3000 xg and 4° C (SORVALL®RC 3B PLUS). The supernatant was transferred to an 1000 ml Erlenmeyer flask and the media was filtered through a 15 µm glass fibre prefilter and a 8 µm mainfilter (Millipore) by using a peristaltic pump at 20 rpm. The filtrate was collected in an 1000 ml Erlenmeyer flask. After the filtration 50 µl endoglycosidase F1 [2 mg/ml] were added to 500 ml filtrate. The mixture was incubated over night at 25° C with low shaking at 60-70 rpm. The next day, the medium was filtered again through a 0.45 µm filter (Millipore).

### 3.1.8 Purification

#### 3.1.8.1 Nickel affinity chromatography

The first purification step was performed on a $Ni^{2+}$ -chelating sepharose column using an Äkta Prime System (Amersham Pharmacia Biotech). The deglycosylated culture supernatant was loaded on the column at a flow rate of 5 ml/min and the flow through was collected. The column was then washed with binding buffer (10 mM Imidazole, 10 mM $Na_2HPO_4$, 10 mM $NaH_2PO_4$, 50 mM NaCl) until a baseline was reached. After an additional wash step with 25 ml wash buffer (50 mM Imidazole, 10 mM $Na_2HPO_4$, 10 mM $NaH_2PO_4$, 50 mM NaCl) the column bound protein was eluted with 30 ml elution buffer (500 mM Imidazole, 10 mM $Na_2HPO_4$, 10 mM $NaH_2PO_4$, 50 mM NaCl). The eluted 1 ml-fractions were analyzed on a 15 % SDS-PAGE (Coomassie- stained).
The fractions that contained VEGF-E were pooled and concentrated up to a volume of 500 µl in 6 ml VIVASpin concentrator, 10 kDa MWCO at 4° C and 3000 xg (Eppendorf Centrifuge 5810R)

#### 3.1.8.2 Gel filtration (Size exclusion chromatography)

This size exclusion chromatography was performed on a Superdex 200 column (Amersham Pharmacia Biotech). The column was first equilibrated with buffer (50 mM Tris/HCl pH 7.5, 100 mM NaCl) at a flow rate of 0.5 ml/min. The concentrated sample was filtered through a 0.22 µm eppendorf filter device and loaded onto the column using a 500 µl loop. Fraction collection (0.5 ml fractions) started 7 ml after injection.

### 3.1.9 Competitive Binding Assays

Canine VEGF-$A_{164}$ (this is the protein corresponding to human VEGF-$A_{165}$) was iodinated with the IODO-GEN method using 0.5 mCi of Na $^{125}I$ (Amersham Pharmacia Biotech, IMS-30) activated for 6 min at RT in a tube coated with 50 µg of Iodogen (Pierce) and mixed with 10 µg of deglycosylated VEGF-$A_{164}$ for 9 min at RT. Reaction was stopped with 1 mM DTT. The iodinated protein was then separated from free iodine on a PD10 gel filtration column (Amersham Pharmacia Biotech) that was equilibrated with 0.5% BSA, 1 mM citric acid in PBS. The resulting specific radioactivity was 85'260 c.p.m./ng. Porcine aortic endothelial (PAE) cells expressing

VEGFR-1 or VEGFR-2 were grown overnight to subconfluency in 24-well tissue culture plates and incubated for 4 hours on ice with 0.1 nM $^{125}$I-labeled VEGF-$A_{164}$ in the presence or absence of the indicated amount of cold competitor. Each concentration point was done in triplicates. Unbound radioactivity was eliminated by 3 washes with MEM, 25 mM HEPES, w/o L-glutamine (Amined) containing 0.25% BSA. Cells were lysed in 200 µl 1 M NaOH and the associated radioactivity determined in a Beckman γ-counter.

### 3.1.10 Crystallization and X-Ray diffraction experiments

Crystallization was performed at 20° C using the hanging-drop vapor-diffusion method. The drops were prepared by mixing 1 µl of the protein (20 mg/ml in 50 mM Tris/HCl, 100 mM NaCl) and 1 µl of the reservoir solution. Each reservoir contained 200 µl of the precipitant solution. For the first crystallization screen the Crystallization Basic Kit for Proteins (SIGMA®) was used, which contains 50 different precipitant solutions.

For X-Ray diffraction experiments, crystals were equilibrated stepwise in cryoprotectant solution (Table 5) and flash-frozen directly in a liquid-nitrogen cryo stream. The measurements were performed either on a in house X-Ray source or at the SLS.

**Table 5** Crystallization buffers and cryoprotectants

| Precipitant solution | Cryoprotectant |
|---|---|
| 0.2 M Li-Sulfate<br>0.1M Tris-HCl pH 8.5<br>30% PEG 4K | + 5 - 20 % Glycerol |
| 0.1 M HEPES pH 7.5<br>0.8 M Na-K-Tartrate | + 5 - 40 % Glycerol |
| 0.1 M Na-citrate pH 5.6<br>20 % Isopropanol<br>20 % PEG 4K | direct, no cryoprotectant needed |
| 0.1 M HEPES pH 7.5<br>10 % Isopropanol<br>20 % PEG 4K | direct, no cryoprotectant needed |

## 3.2 VEGFR-2

### 3.2.1 Cloning of VEGFR-2_D1-3_C-GST by PCR-Gateway-Technology

**Table 6** Amplifying of VEGFR-2_D1-3: an *att*B-product

| | Sample Reaction | Positive Control Reaction | Negative Control Reaction |
|---|---|---|---|
| template<br>50 ng | 1.5 µl<br>(pPICZα_D1-3) | 1.5 µl<br>(pPICZα_D1-3) | 1.5 µl<br>(pPICZα_D1-3) |
| primer forward (1:20)<br>125 ng | 1 µl | 1 µl (D2-3) | -- |
| primer reverse (1:20)<br>125 ng | 1 µl | 1 µl (D2-3) | -- |
| dNTPs [10 mM] | 1 µl | 1 µl | 1 µl |
| buffer 5x<br>F-518 (FINNZYMES) | 10 µl | 10 µl | 10 µl |
| $H_2O$ | 35 µl | 35 µl | 37 µl |
| polymerase phusion<br>(F-530L, FINNZYMES) | 1 µl | 1 µl | 1 µl |
| **Total** | **50 µl** | **50 µl** | **50 µl** |

To amplify VEGFR-2_D1-3, a pPICZα_D1-3 vector was used as template. The primers for this reaction were specifically designed. The following primers were used (Microsynth):

Forward primer:
5'-
GGGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGGCCTCTGTGGGTTTGCCTAGT
G-3'
71 nucleotides ATTB1 SD/ KOZAK D1

Reverse primer:
5'- GGGGACCACTTTGTACAAGAAAGCTGGGTCTCCTTGGAAGTATAAGTTTTCTTTTTCATGGACCCTGAC-3'
69 nucleotides ATTB2 TEV D3 REV

**Table 7** PCR-Conditions

| Segment | Cycles | Temperature | Time |
|---|---|---|---|
| **1** | 1 | 98°C | 2' |
| **2** | 20 | 98°C | 30" |
| | | 55°C | 30" |
| | | 72°C | 40" |
| **3** | 1 | 72°C | 2' |
| | hold | 10°C | |

### 3.2.2 BP Recombination Reaction

A BP recombination reaction was performed between an *att*B-flanked DNA fragment and an *att*P-containing donor vector to generate an entry clone.

**Table 8** Scheme of BP Recombination Reaction

| | Sample | Positive Control |
|---|---|---|
| attB-PCR product (100ng) | 0.5 µl | -- |
| pDONR™ vector (150ng/µl) | 0.5 µl | 0.5 µl |
| pEXP7-tet positive control (50ng/µl) | -- | 0.5 µl |
| BP Clonase™ Reaction Buffer 5x | 1 µl | 1 µl |
| TE-buffer, pH 8.0 | 2 µl | 2 µl |
| BP Clonase™ enzyme mix | 1 µl | 1 µl |
| **Total** | **5 µl** | **5 µl** |

pDONR™ vector, pEXP7-tet positive control, BP Clonase™ Reaction Buffer 5x and BP Clonase™ enzyme mix were purchased from the company Invitrogen®.

The reaction was incubated in the thermocycler over night at 25°C. 0.5 µl Proteinase K (Invitrogen®) were added and the mix was incubated for 15 min at 37° C.

Electroporation in *E.coli*, Minipreps, Minicultures and Restriction digest then followed as described in 3.1.2 – 3.1.4 (p. 9).

### 3.2.3 LR Recombination Reaction

A LR recombination reaction was performed between an *att*L-containing entry clone and an *att*R-containing destination vector to generate an expression clone.

**Table 9** Scheme of the LR Recombination Reaction

| | Sample | Positive Control |
|---|---|---|
| entry clone 20-30ng | 2 µl | -- |
| destination vector (pDEST24), 100ng | 0.75 µl | 0.75 µl |
| pENTER™-gus | -- | 1 µl |
| 5x LR Clonase™ Reaction Buffer | 1 µl | 1 µl |
| TE Puffer, pH 8 | 0.25 µl | 1.25 µl |
| LR Clonase™ enzyme mix | 1 µl | 1 µl |
| **Total** | **5 µl** | **5 µl** |

5x LR Clonase™ Reaction Buffer und LR Clonase™ enzyme mix were purchased from the company Invitrogen®. As entry clone the product of the BP recombination reaction was used.

The reaction was incubated in the thermocycler over night at 25°C for. 0.5 µl Proteinase K (Invitrogen®) were added and the mix was incubated for 15 min at 37° C.

Electroporation in *E.coli*, Minipreps, Minicultures and Restriction digest then followed as described in 3.1.2 – 3.1.4 (p. 9).

### 3.2.4 Chemical Transformation into *E.coli*

1 µl LR reaction-mix was added to 75 µl DH5α cells and incubated on ice for 30 min. After 45 seconds heat shock at 42° C and 2 min incubation on ice 200 µl SOC-Medium were added to the mixture, which was further incubated at 37° C for 30 min to allow expression of Ampicillin resistance. 100 µl of cell suspension were then spreaded on LB agar plates with Ampicillin [50 µg/ml] and incubated over night at 37° C.

### 3.2.5 Protein expression

Small-scale expression:

To find out the optimal point to harvest the cells, first a small-scale expression was made. Therefore 5 ml of LB media with antibiotics were inoculated and grown over night at 37° C and 200 rpm. The next day, 0.5 ml of these suspensions were diluted in 5 ml fresh LB media with antibiotics. The cultures were grown (37° C and 200 rpm) until they reached an $OD_{600}$ of 0.6 -1.0. Expression was then induced with 1 mM IPTG. 0.5 ml samples were collected every hour and accordingly analyzed on a 10 % SDS-PA-gel and with Western Blot.

Large-scale expression:

50 ml of LB media with antibiotics were inoculated and grown over night at 37° C and 200 rpm. The next day, the culture was diluted to 1 l LB media and incubated at 37° C and 200 rpm to reach an OD of 0.6-1.0. To induce expression 1 mM IPTG was added and the cultures were incubated for 2-4 hours (depending on the *E.coli* strain) at 30° C.

### 3.2.6 Dot Blot and Western Blot

Dot Blot (Test for expression and solubility):

250 µl cell suspension were added to 3 ml autoinduction medium (ZYP-5052 after F.W. Studier) in 10 ml 24-deepwell plates and incubated over night at 37 °C and 200 rpm. The next day for each cell suspension a whole cell lysate and a soluble sample was prepared and filtrated through a PVDF-membrane using a standard lab protocol.
After this the membrane was blocked with Qiagen blocking buffer for 30 min and incubated with an antibody-mix for 1 hour. The first antibody was a polyclonal rabbit anti-GST-IgG (100 µg/ml, SantaCruz Biotechnology) and the second one a monoclonal goat-anti-rabbit-AP-IgG (200 µg/0.5 ml). The membrane was washed with TBS-buffer (20 mM Tris/HCl pH 7.5, 150 mM NaCl) and TTBS-buffer (20 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.05 % Tween 20) and developed with NBT/BCIP in AP-substrate buffer (100 mM Tris/HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$).

Western Blot:

A 10 % SDS-PA-gel was run with 30 mA for 1 hour. Then the gel was blotted on a PVDF-membrane with 60 mA for 2 hours using a semi-dry transfer buffer system (Bio rad). After blotting the membrane was processed as described in the Dot Blot procedure.

### 3.2.7 Preparation of cell lysates

The frozen pellet from 1 l *E.coli* was resuspended in 24 ml ice cold lysis buffer (50 mM Tris/HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 10 % glycerol, 1 mM PMSF, 1 mM DTT). 1.25 ml 20 % Triton-X100 was added to final 1 %. Accordingly, 2-5 mg lysozyme was added and the mix was left on ice for 1 hour and then sonicated on ice for 30 seconds (0.5'' on/0.5'' off, macrotip 65 %). The cell debris were separated by centrifugation (27'000 xg; 4° C; 30 min) and the supernatant was used for purification.

### 3.2.8 Purification by Glutathione Sepharose (affinity chromatography)

The clear lysate was applied twice to the glutathione-sepharose-column by gravity flow and the flow through was collected. The column was washed with 50 ml wash buffer (50 mM Tris/HCl pH 8.0, 1 M NaCl, 5 mM EDTA, 10 % glycerol, 0.1 % Triton X-100, 1 mM DTT) and the wash fraction was collected. 15 ml of elution buffer (50 mM Tris/HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 10 % glycerol, 1 mM DTT, 1 mM reduced glutathione) were added to the column to elute the protein and 1 ml fractions were collected. The fractions were analyzed on a 10 % SDS-PAGE.

# 4 RESULTS

## 4.1 VEGF-E-L13

A VEGF-E-L13-mutant was cloned and transformed into the yeast *P. pastoris* for expression as a secreted protein. The protein was purified by nickel affinity and size exclusion chromatography. The purified protein was used for binding assays and crystallization screens.

### 4.1.1 Cloning of VEGF-E-L13-Mutant by Site Directed Mutagenesis

Positive VEGF-E-L13 clones were analyzed by restriction digest with the restriction enzymes Afl III and Eco24I. Compared to the template DNA (pPICZαA-VEGF-E-L1) of the cloning reaction the pPICZαA-VEGF-E-L13 shows a different restriction pattern.
Figure 3 shows the result of a restriction digest with different positive VEGF-E-L13 clones and as negative control VEGF-E-L1 on a 1 % agarose gel. The restriction enzyme Eco24I linearizes VEGF-E-L13 by cutting at only one side (3832 bp), while it cuts VEGF-E-L1 twice (2601 bp, 1231 bp). This indicates that the mutation of loop-3 in VEGF-E was successful. For the restriction enzyme Afl III no difference was observed between this two VEGF-E-loop-mutants. This enzyme was only used to exclude all sorts of unreasonable mutants.
The clone S3 shows the expected restriction pattern. The correct sequence of clone 3 was confirmed by DNA sequencing (Microsynth).

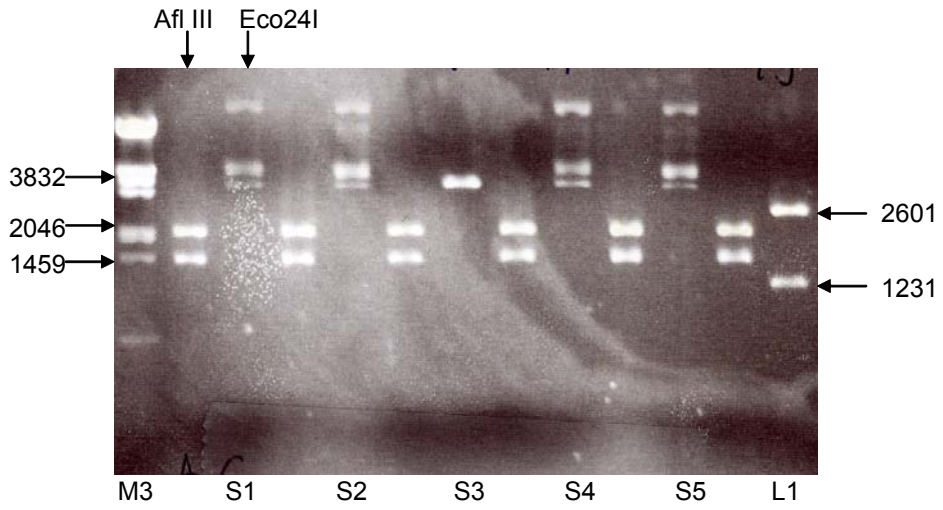


**Figure 3.** *Restriction digest of VEGF-E-L13.* This picture of an 1% agarose-gel shows the result of a restriction digest of different presumable VEGF-E-L13 clones with the restriction enzymes Afl III and Eco24I. Clone S3 shows the expected restriction pattern: it was cleaved twice with the enzyme Afl III (bands of 2046 bp and 1459 bp) and once with Eco24I (3832 bp)

### 4.1.2 Protein expression and purification

#### 4.1.2.1 Nickel affinity chromatography

Small-scale expression was performed to select for high-expression clones. Out of a 100 ml test expression (*P. pastoris*) 2 mg of pure protein were obtained. The purified protein was analyzed by SDS-PAGE. VEGF-E-L13, which forms a disulfide linked homodimer in solution, was detected as a clear band at about 15 kDa under reducing condition and at about 30 kDa under non-reducing conditions (Figure 4). The fractions containing the VEGF-E-L13 were pooled and 4 ml of PBS were added to dilute the Imidazole concentration. The pooled fractions were then concentrated to 15 mg/ml. The protein was used for binding analysis

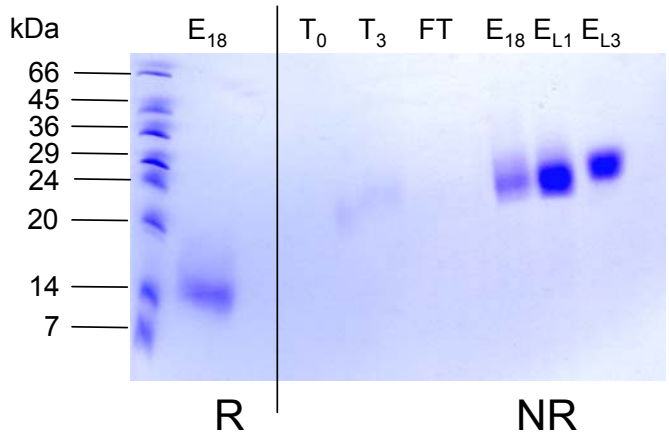


**Figure 4.** *Production and purification of VEGF-E-L13 by nickel affinity chromatography.* Left panel R: $E_{18}$ is one of the eluted fractions after the $Ni^{2+}$-column. The protein forms a monomer of about 15 kDa under reducing conditions (R). Right panel: The protein forms a dimer of about 30 kDa under non-reducing conditions (NR). $T_0$ is the time point before induction and T3 is the time point when the production was stopped (supernatant which was loaded on the column). FT (flow-through) is the fraction which did not bind to the column. As a control two other VEGF-E-loop-mutants were loaded on the gel ($E_{L1}$ and $E_{L3}$).

### 4.1.2.2 Size exclusion chromatography (Gel filtration)

The fractions containing pure VEGF-E-L13 were pooled, concentrated and purified by gel filtration. The protein eluted as a homogenous peak at 15.6 ml from the Superdex 200 column, which corresponds to an apparent molecular size of about 37 kDa. The peak at 11.7 ml corresponds to a molecular size of about 250 kDa and most likely represents some oligomers of the protein.

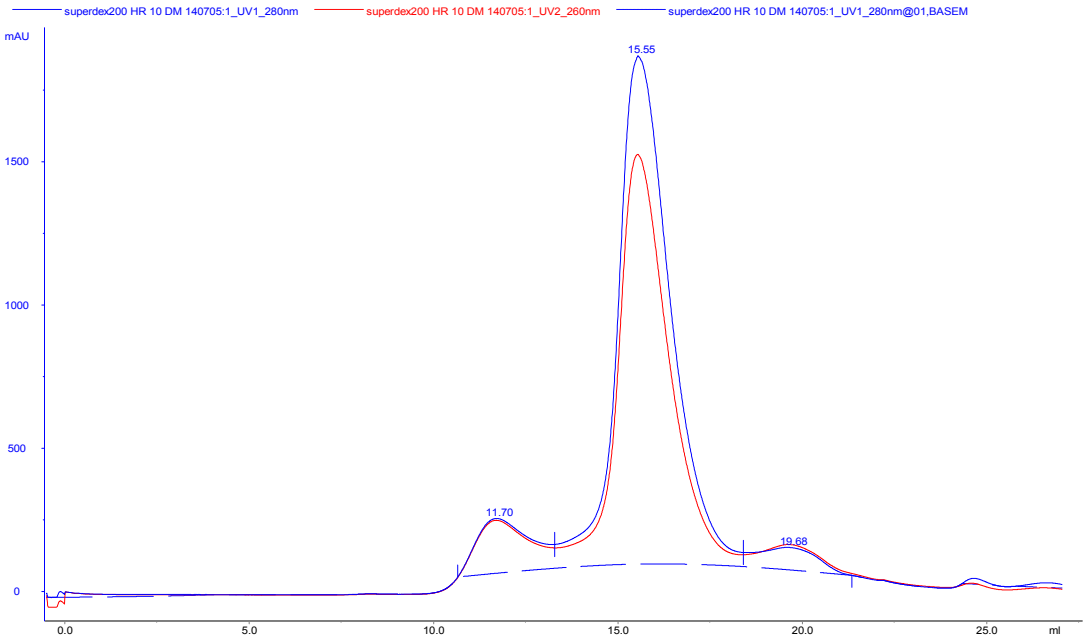


**Figure 5.** *Size exclusion chromatography of VEGF-E-L13 on a Superdex 200 column.* VEGF-E-L13 eluted as a homogenous peak at 15.6 ml, which corresponds to an apparent molecular size of about 37 kDa. The blue line shows the absorbance at 280 nm and the red line the absorbance at 260 nm.

The fractions were analyzed on a 15 % SDS-PA-gel (Figure 6). The VEGF-E-L13 protein forms a monomer under reducing conditions, which has the size of about 15 kDa. The purity of the protein was high enough to proceed with crystallization experiments. Therefore the fractions E11-E15 were pooled and concentrated to 20 mg/ml. From a 500 ml culture 10 mg of highly pure VEGF-E-L13 were obtained.

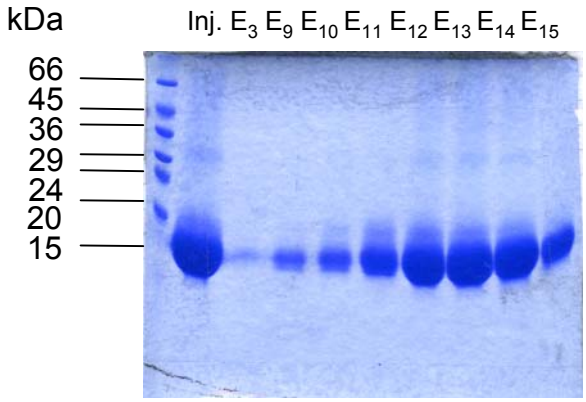


**Figure 6.** *Gel filtration of VEGF-E-L13.* Coomassie-stained 15 % SDS-PA-gel showing the injected sample (Inj.) and the eluted fractions of VEGF-E-L13 (E3-E15) after the gel filtration.

### 4.1.3 Competitive Binding Assays

Competitive binding assays were performed on PAE cells expressing either VEGFR-1 or -2. Binding of radiolabeled VEGF-A to VEGFR-2 was competed with VEGF-E, but not with the loop mutants VEGF-E-L1, VEGF-E-L3 and VEGF-E-L13 or PlGF. This shows that exchanging either one or both of these loops abolished binding to VEGFR-2 (Figure 7). Interestingly, competition with the point mutant VEGF-E-R46I was more efficient than with the native viral protein (VEGF-E) indicating that during Orf virus evolution VEGF-E diverged from optimal receptor binding.
When these mutants were tested on VEGFR-1 expressing cells, only VEGF-E-L13 showed specific binding besides VEGF-A and PlGF. The results of these experiments indicate that a suitable set of the loops L1 and L3 determines receptor specifity.

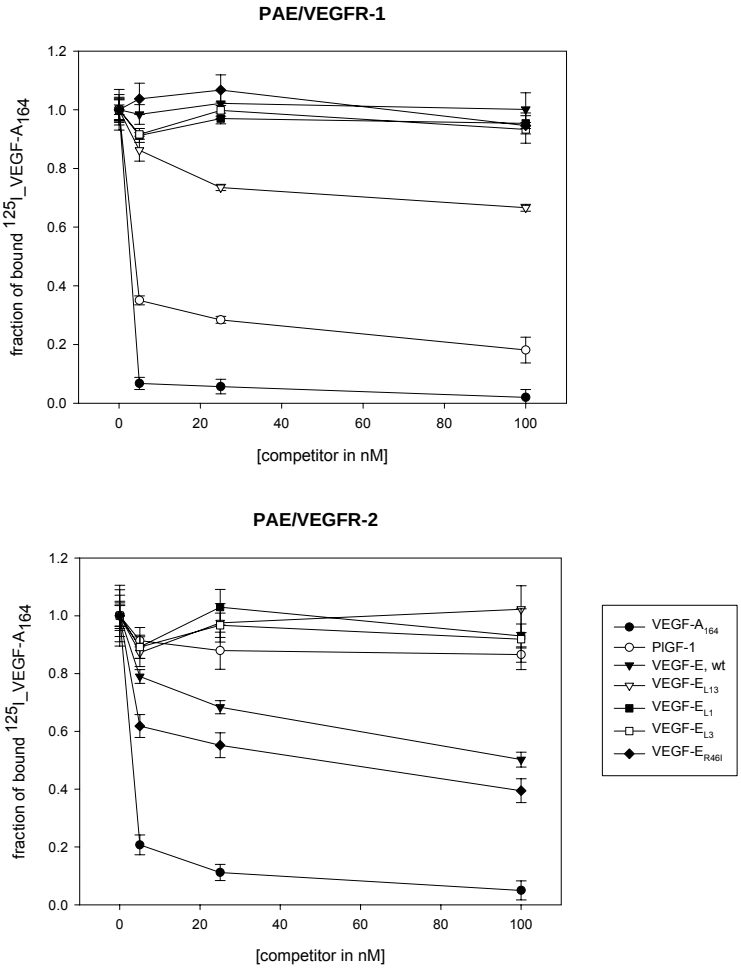


**Figure 7.** *Competitive Binding Assay with 0.1 mM $^{125}$I-VEGF-A on PAE cells expressing either VEGFR-1 or -2.* Upper panel: VEGFR-1 expressing PAE cells. The VEGF-E-L13 mutant shows an affinity to VEGFR-1. Lower panel: VEGFR-2 expressing PAE cells. All the loop-mutants lost affinity to VEGFR-2. Competition with the point mutant VEGF-E-R46I was more efficient than with the wildtype VEGF-E (wt).

### 4.1.4 Crystallization of VEGF-E-L13 and preliminary X-Ray diffraction

The purified and concentrated VEGF-E-L13 mutant was used to set up initial crystallization screens using the Crystallization Basic Kit (SIGMA) and both the known crystallization conditions for wildtype VEGF-E and PlGF. Four hits were obtained from the Sigma Kit and were used as starting conditions for further optimization (Figure 8 and 9, left panel). In all conditions the crystals appeared as thin needles or needle bundles. Extensive screens were setup to optimize crystal growth by varying precipitant concentration against pH, and by varying the temperature. However, no improvement of crystal quality could be achieved.

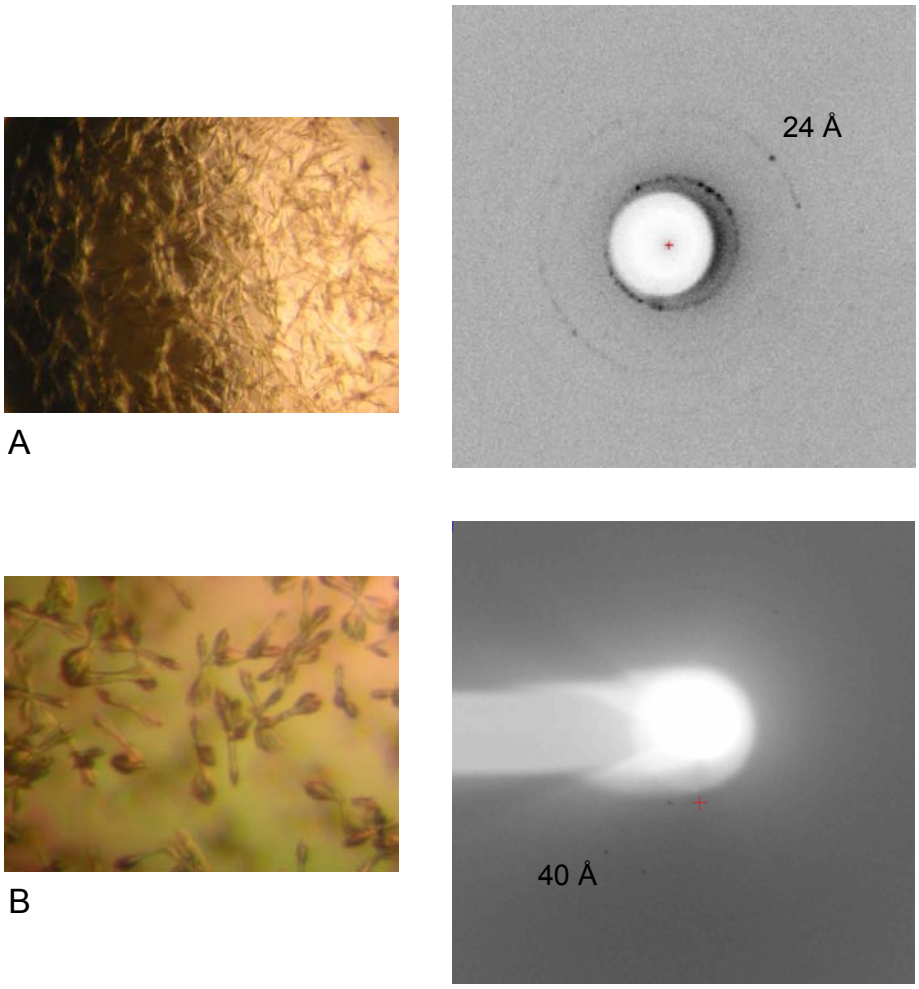


**Figure 8.** *First crystallization screen (SIGMA Basic Screen Kit) and X-Ray diffraction.* The following conditions were used: for sample A 0.2 M Li-sulfate, 0.1 M Tris-HCl pH 8.5, 30 % PEG 4 K and for the measurements, performed on a in house X-Ray source, as cryoprotectant 5 – 20 % glycerol was added to the precipitant solution. For sample B the precipitant solution contained 0.1 M HEPES pH 7.5, 0.8 M Na-K-Tartrate and as cryoprotectant 5 – 40 % glycerol. The measurement were performed at the SLS.

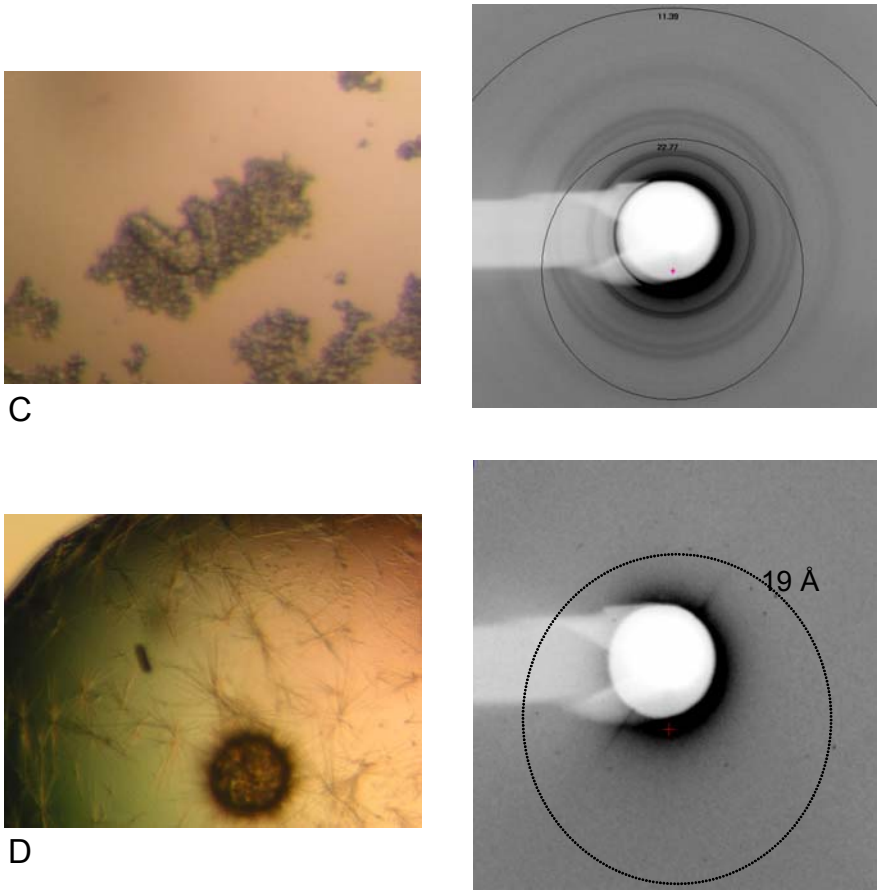


**Figure 9.** *First crystallization screen (SIGMA Basic Screen Kit) and X-Ray diffraction.* The following conditions were used: for sample C 0.1 M Na-citrate pH 5.6, 20 % PEG 4K, 20 % Isopropanol and for sample D the precipitant solution contained 0.1 M HEPES pH 7.5, 20 % PEG 4K, 10 % Isopropanol. Both measurements were performed at the SLS.

For X-Ray diffraction experiments, crystals were equilibrated stepwise in cryoprotectant solution if needed and flash-frozen directly in a liquid-nitrogen cryo stream. The measurements were performed either on a in house X-Ray source or at the SLS (Figure 8 and 9, right panel). Poor diffraction was observed for all the crystals. However, from the PEG 4K/ Isopropanol condition at pH 7.5 some reflections were detected up to 19 Å resolution (Figure 9 D). The PEG/ Isopropanol condition grown at pH 5.6 (Figure 9 C) yielded a series of rings to 20 Å resolution on the diffraction image, indicating a random orientation of the molecules in the crystals. However these results clearly indicated the presence of protein crystals.

## 4.2 VEGFR-2

The VEGFR-2_D1-3-GST construct was cloned and transformed to different *E.coli* strains for expression. Test for expression and solubility were made with different DNA samples. The protein was purified by glutathione sepharose affinity chromatography.

### 4.2.1 Cloning of VEGFR-2_D1-3_GST

To verify the identity of different positive VEGFR-2_D1-3 clones a restriction digest was made. Three VEGFR-2_D1-3 clones were digested with the restriction enzymes Afl III and Eco24I. Compared to the template DNA (pDEST 24), which contains three Afl III (3069 bp, 2838 bp and 1054 bp) and two Eco24I (6947 bp and 14 bp), the expression clone (pDEST24_D1-3_GST) contains three Afl III restriction sites (2838 bp, 2636 bp and 798 bp) and one Eco24I restriction site (6258 bp).

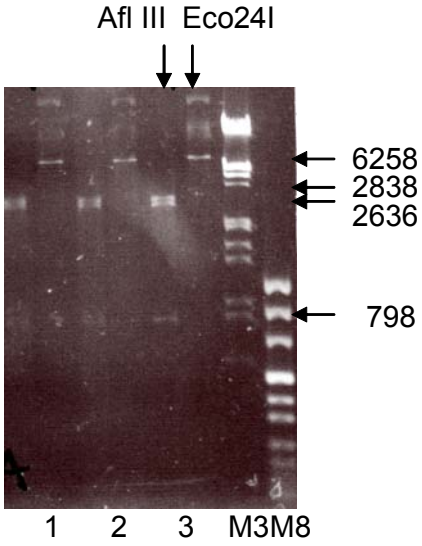


**Figure 10.** *Restriction digest of VEGFR-2_D1-3_GST*. This picture of an 1% agarose- gel shows the result of a restriction digest of different positive VEGFR-2_D1-3_GST clones. As restriction enzymes Afl III and Eco24I were used. The clones 2 and 3 show the expected restriction pattern: they were cleaved three times with the enzyme Afl III (bands of 2838 bp, 2636 bp and 798 bp) and once with Eco24I (6258 bp).

### 4.2.2 Test of expression and solubility (Dot Blot)

To find out if expression works and if the proteins are soluble or not two different DNA samples were transformed to two different *E.coli*-strains and were grown in a small volume culture. Cells were lysed with a chemical method, applied on a PVDF-membrane (Dot Blot) and detected with α-GST-antibodies.

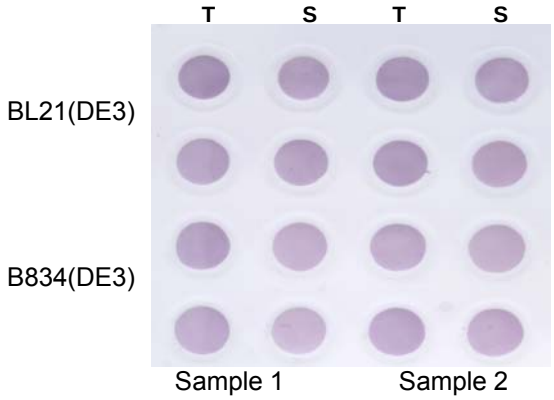


**Figure 11.** *Expression and solubility screen with two VEGFR-2_D1-3-GST-DNA samples.* There are two different VEGFR-2_D1-3-DNA samples transferred to two different *E.coli*-strains. The expression test shows that both of this *E.coli*-strains express the protein and there is a lot of soluble protein.

The expression and solubility test showed that expression occurred and that most of the protein is soluble. However, no conclusion could be drawn whether the signal was generated by the intact fusion protein of interest, or by fragments or other proteins (Figure 11). Western blot analysis of the same samples showed that the signal was caused by degradation products of the protein (Figure 12).

To better control the time of induction and the protein degradation a small-scale expression was performed. Expression was induced with IPTG and samples were taken every hour for Western Blot analysis (Figure 12). A signal for the full-length fusion protein of about 60 kDa was only detectable after 2 hours induction. Longer induction time resulted in no signal for the full length protein.

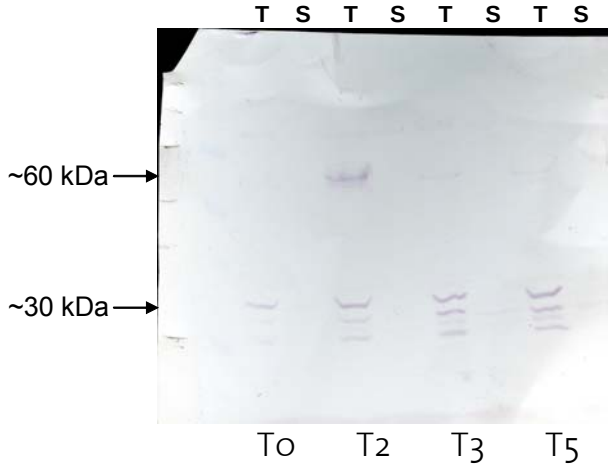


**Figure 12.** *Western Blot with different time points after small-scale expression of VEGFR-2_D1-3_GST in E.coli (BL21pLysS (DE)).* The full-length fusion protein of about 60 kDa is only detectable after 2 hours induction. Later time point show only the degradation products.

### 4.2.3 Protein expression and Purification of VEGFR-2_D1-3-GST

A large-scale production of VEGFR-2_D1-3-GST was performed following the results from the small-scale expression. Protein purification was performed by glutathione sepharose (affinity chromatography) and the eluted fractions were analyzed on a 10% SDS-PA-gel (Figure 13).
For the whole protein (D1-3 and GST-tag) a molecular size of about 60 kDa was expected. None of these fractions showed a band in this range, but all of them contained degradation products in the range of about 30 kDa.

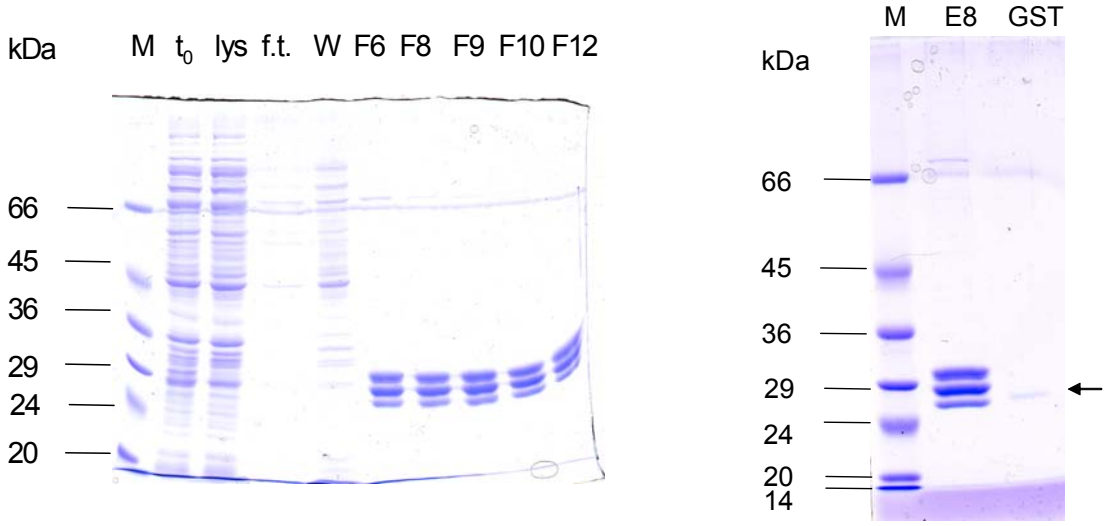


**Figure 13.** *Production and purification of VEGFR-2_D1-3_GST by Glutathione sepharose.* Left: 10 % SDS-PA-gel as a result of the production and purification of VEGFR-2_D1-3_GST out of 1000 ml culture (*E.coli*). $T_0$ time point before inducing expression, lys =lysate, f.t. = flow through, W = wash fraction and F6-F12 are the eluted fractions. Right: On this 10 % SDS-PA-gel there is one of the eluted fractions after glutathione sepharose column to see and as a control the GST-protein.

All three degradation products are in a range of about 30 kDa. Since this is half the size of the expected full-length fusion protein, the question arose, whether two of the three bands would contain the two halfs of the molecule. Therefore the three bands were plotted on a PVDF-membrane, stained by Amidoblack and sent for Edman-Sequencing. The sequencing was only possible for the upper band (MKKFLST) which contained the 43 C-terminal residues of domain 3 fused to GST. The middle band was blocked, and the lowest band could not be sequenced.

# 5 DISCUSSION

The aim of the diploma thesis was to generate the loop chimera VEGF-E-L13, to characterize its binding properties to VEGFR-1 and –2 and to crystallize the mutated protein. This protein was designed based on the observed differences in the loop regions 1 and 3 between the crystal structures of VEGF-E and PlGF with the aim to clarify the role of these loops in receptor VEGFR-specificity.
Cloning was performed by SiteDirected Mutagenesis using a VEGF-E-L1 mutant as template. The yeast *Pichia pastoris* was chosen as expression system as it has been successfully used for the expression of related VEGF's in the lab. The protein was designed with a cleavable aminoterminal hexahistidine tag to allow a simple two step purification by nickel affinity and size exclusion chromatography.
For competitive binding assays the nickel affinity purified protein was used. Competitive binding assays on PAE cells expression either VEGFR-1 or –2 with radiolabeled VEGF-$A_{164}$ were performed with the VEGF-E-L13, with the individual loop mutants L1 and L3, and the point mutant VEGF-E-R46I, which were cloned in the course of my semester thesis. Binding of the radiolabeled VEGF-A to VEGFR-2 was competed with VEGF-E, but not with the loop mutants VEGF-E-L1, VEGF-E-L3, VEGF-E-L13 or PlGF. Competition with the point mutant VEGF-E-R46I was more efficient than with the native viral protein (VEGF-E) yet did not establish binding to VEGFR-1, suggesting that Arg46 does not directly regulate interaction with VEGFR-1, but is critical for proper binding to VEGFR-2. An important finding of this studies showed the results of VEGF-E-L13 mutant: if both loops of VEGF-E were exchanged by sequences from PlGF binding to VEGFR-2 got lost but the mutant bound, although with lower affinity than VEGF-A and PlGF, to VEGFR-1. This indicates that L1 and L3 interact which each other and that the relative orientation between L1 and L3 determines VEGFR-specificity.
To know more about the orientation of these two loops crystallization of VEGF-E-L13 was performed. The purified and concentrated VEGF-E-L13 mutant was used to set up initial crystallization screens using the Crystallization Basic Kit (SIGMA) and both the known crystallization conditions for wildtype VEGF-E and PlGF. Four hits were obtained from the Sigma Kit and were used as starting conditions for further optimization. For X-Ray diffraction experiments, these crystals were equilibrated stepwise in cryoprotectant solution and flash-frozen directly in a liquid-nitrogen cryo stream. The measurements were performed either on a in house X-Ray source or at the SLS. Because these crystals were very small they only diffracted to low resolution.
To get bigger crystals the four conditions of the Sigma Kit were optimized by varying the pH, precipitant concentration and temperature. These optimization attempts resulted either in large amounts of phase separation in the drops or in very thin needles. Apparently the protein is prone to preferentially grow in one dimension. To avoid phase separation an additive screen using 24 different detergents was set up, however, little improvement was achieved. Triton-X100 showed the best results, so it would be advisable to perform all purification steps and repeat the Sigma basic screen in the presence of Triton-X100.
Microseeding experiments were also performed to improve crystal quality. Therefore, a 2 µl drop containing VEGF-E-L13 needles was homogenized with a small glass rod to prepare a seed stock. These seeds were added to a freshly prepared crystallization drop to induce crystal growth. However, no crystals could be grown from these setups.
The purified VEGF-E-L13 protein contained an N-terminal His-tag which most likely effected the proper crystallization of the protein. In a future experiment it would be

advisable to remove the tag before setting up crystallization to see if differences could be observed in crystal growth.

To understand the specific interaction of VEGF-receptors with their ligands, the crystal structures of receptor-ligand complexes are needed in addition to the structural information about the individual ligands. An optional project for this diploma thesis was to clone a VEGFR-2 fragment that contains the first three Ig-domains fused to a GST for future co-crystallization of ligand-receptor complexes. Expression of selected VEGFR domains in eukaryotic systems is in progress in the lab, however low yields of expressed protein are obtained and production time is much longer compared to *E. coli*. The aim of this additional project was to assess *E. coli* as an alternative expression system for fast production of suitable amounts of protein that could be used for complex formation either with the wildtype ligands VEGF-E or VEGF-A, or with the mutant VEGF-E-R46I.
Expression and solubility tests were made with positive transformants in different *E.coli* strains. The results of the dot blots indicated the presence of good amounts of protein in the soluble form, but did not allow any conclusion about the integrity of the expressed protein. Additional analysis by SDS-PAGE and Western Blot revealed that proteolytic degradation occurred during expression: Three fragments in the range of 30 kDa were detected instead of the expected 60 kDa. Only the largest of these three fragments could be sequenced (Edman-Sequencing) and corresponded to the carboxyterminal 43 residues of VEGFR-2-domain 3 fused to GST. Based on a homology model of domain 3 the cleavage occurred in the middle of a β-sheet, indicating that the protein most likely was not proper folded and therefore more sensitive to proteases. Clearly more expression tests in different *E.coli* strains with different genotypes need to be performed, to find a strain that shows less degradation. Alternatively the known cleavage sites could be mutated by site directed mutagenesis. Since we do not know how many cleavage sites are present upstream the MKKFLST, this would only be considered in case no more or only a few cleavage sites are present.
Many other approaches could be tested, e.g. including an additional tag at the amino terminus, trying different tags, production in inclusion bodies and refolding. However, this case clearly demonstrates that *E.coli* is not always suitable for protein expression, although it is fast, easy to handle and cheaper than eukaryotic expression systems.

# 6 ABBREVIATIONS

| | |
|---|---|
| AP | Alkaline phosphatase |
| BCIP | 5-Bromo-4-Chloro-3-Indolylphosphatyl-p-Toluidine Salt |
| BSA | Bovine Serum Albumine |
| BMGY | Buffered Glycerol-complex Medium |
| BMMY | Buffered Methanol-complex Medium |
| c.p.m. | counts per minute |
| DTT | Dithiothreitol |
| EDTA | Ethylenediaminetetraacetic acid |
| IPTG | Isopropyl-beta-D-thiogalactopyranoside |
| LB | Luria-Bertani media |
| MEM | Minimal essential medium |
| MWCO | Molecular weight cut off |
| NBT | Nitro-blue Tetrazolium Chloride |
| PAE | Porcine aortic endothelial |
| PAGE | Polyacrylamid Gel Electrophoresis |
| PBS | Phosphate Buffered Saline |
| PEG | Polyethylene Glycol |
| PMSF | Phenylmethylsulphonylfluoride |
| RT | Room temperature |
| SDS | Sodium Dodecyl Sulfate |
| VEGF | Vascular Endothelial Growth Factor |
| VEGFR | Vascular Endothelial Growth Factor Receptor |
| YPD | Yeast Extract Peptone Dextrose Medium |

# 7 LITERATURE

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